IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of
HENNEQUIN *et al.* Group Art Unit: 1624
Appln. No.: 09/806,836 Examiner: Truong
Filed: 12th June 2001
FOR: CHEMICAL COMPOUNDS

DECLARATION

STEPHEN ROBERT WEDGE declares:-

1. That he holds the degrees of BSc (Hons) and Ph.D. from the University of Keele, Staffordshire, UK and that he is a Bioscientist and Associate Director in the Cancer Discovery Department of ASTRAZENECA UK Limited.

2. That he has worked for the past 17 years in the fields of biochemistry and cancer pharmacology, developing and evaluating novel therapeutic strategies for the treatment of cancer.

3. That he was a Lead *In Vivo* Bioscientist on the project which requested the testing of the compounds exemplified in US Patent Application No. 09/806,836.

4. That biologists working under his direction have tested two compounds C1 and C2 of US Patent 6,645,969 to determine their activity, *in vitro*, as inhibitors of the tyrosine kinase activity of the VEGF receptors Flt1 and KDR, as well as to determine their corresponding activity in a HUVEC proliferation assay.

The compounds C1 and C2 are as follows:

C1 is 4-(3-chlorophenoxy)-6,7-dimethoxyquinazoline, and
C2 is 4-(3,4,5-trimethoxyphenoxy)-6,7-dimethoxyquinazoline.

And that biologists working under his direction have tested the compounds of the Examples of the subject application, *inter alia*, to determine their activity, *in vitro*, as inhibitors of the tyrosine kinase activity of the VEGF receptors Flt1 and KDR, as well as to determine their corresponding activity in a HUVEC proliferation assay, which data is presented on the

Table attached to this declaration as APPENDIX I.

5. That the above-mentioned determinations were effected according to the following test procedures, which are the same as the procedures set forth in the specification of the subject application at pages 42-44, but will be repeated below for convenience of reference:

(a) In Vitro Receptor Tyrosine Kinase Inhibition Test

This assay determines the ability of a test compound to inhibit tyrosine kinase activity. DNA encoding VEGF, FGF or EGF receptor cytoplasmic domains may be obtained by total gene synthesis (Edwards M, International Biotechnology Lab 5(3), 19-25, 1987) or by cloning. These may then be expressed in a suitable expression system to obtain polypeptide with tyrosine kinase activity. For example VEGF, FGF and EGF receptor cytoplasmic domains, which were obtained by expression of recombinant protein in insect cells, were found to display intrinsic tyrosine kinase activity. In the case of the VEGF receptor Flt1 (Genbank accession number X51602), a 1.7kb DNA fragment encoding most of the cytoplasmic domain, commencing with methionine 783 and including the termination codon, described by Shibuya et al (Oncogene, 1990, 5: 519-524), was isolated from cDNA and cloned into a baculovirus transplacement vector (for example pAcYM1 (see The Baculovirus Expression System: A Laboratory Guide, L.A. King and R. D. Possee, Chapman and Hall, 1992) or pAc360 or pBlueBacHis (available from Invitrogen Corporation)). This recombinant construct was co-transfected into insect cells (for example Spodoptera frugiperda 21(Sf21)) with viral DNA (eg Pharmingen BaculoGold) to prepare recombinant baculovirus. (Details of the methods for the assembly of recombinant DNA molecules and the preparation and use of recombinant baculovirus can be found in standard texts for example Sambrook et al, 1989, Molecular cloning - A Laboratory Manual, 2nd edition, Cold Spring Harbour Laboratory Press and O'Reilly et al, 1992, Baculovirus Expression Vectors - A Laboratory Manual, W. H. Freeman and Co, New York). For other tyrosine kinases for use in assays, cytoplasmic fragments starting from methionine 806 (KDR, Genbank accession number L04947), methionine 668 (EGF receptor, Genbank accession number X00588) and methionine 399 (FGF R1 receptor, Genbank accession number X51803) may be cloned and expressed in a similar manner.

For expression of cFlt tyrosine kinase activity, Sf21 cells were infected with

plaque-pure cFlt recombinant virus at a multiplicity of infection of 3 and harvested 48 hours later. Harvested cells were washed with ice cold phosphate buffered saline solution (PBS) (10mM sodium phosphate pH7.4, 138mM sodium chloride, 2.7mM potassium chloride) then resuspended in ice cold HNTG/PMSF (20mM Hepes pH7.5, 150mM sodium chloride, 10% v/v glycerol, 1% v/v Triton X100, 1.5mM magnesium chloride, 1mM ethylene glycol-bis(βaminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1mM PMSF (phenylmethylsulphonyl fluoride); the PMSF is added just before use from a freshly-prepared 100mM solution in methanol) using 1ml HNTG/PMSF per 10 million cells. The suspension was centrifuged for 10 minutes at 13,000 rpm at 4°C, the supernatant (enzyme stock) was removed and stored in aliquots at -70°C. Each new batch of stock enzyme was titrated in the assay by dilution with enzyme diluent (100mM Hepes pH 7.4, 0.2mM sodium orthovanadate, 0.1% v/v Triton X100, 0.2mM dithiothreitol). For a typical batch, stock enzyme is diluted 1 in 2000 with enzyme diluent and 50μl of dilute enzyme is used for each assay well.

A stock of substrate solution was prepared from a random copolymer containing tyrosine, for example Poly (Glu, Ala, Tyr) 6:3:1 (Sigma P3899), stored as 1 mg/ml stock in PBS at -20°C and diluted 1 in 500 with PBS for plate coating.

On the day before the assay 100μl of diluted substrate solution was dispensed into all wells of assay plates (Nunc maxisorp 96-well immunoplates) which were sealed and left overnight at 4°C.

On the day of the assay the substrate solution was discarded and the assay plate wells were washed once with PBST (PBS containing 0.05% v/v Tween 20) and once with 50mM Hepes pH7.4.

Test compounds were diluted with 10% dimethylsulphoxide (DMSO) and 25μl of diluted compound was transferred to wells in the washed assay plates. "Total" control wells contained 10% DMSO instead of compound. Twenty five microlitres of 40mM manganese(II)chloride containing 8μM adenosine-5'-triphosphate (ATP) was added to all test wells except "blank" control wells which contained manganese(II)chloride without ATP. To start the reactions 50μl of freshly diluted enzyme was added to each well and the plates were incubated at room temperature for 20 minutes. The liquid was then discarded and the wells

were washed twice with PBST. One hundred microlitres of mouse IgG anti-phosphotyrosine antibody (Upstate Biotechnology Inc. product 05-321), diluted 1 in 6000 with PBST containing 0.5% w/v bovine serum albumin (BSA), was added to each well and the plates were incubated for 1 hour at room temperature before discarding the liquid and washing the wells twice with PBST. One hundred microlitres of horse radish peroxidase (HRP)-linked sheep anti-mouse Ig antibody (Amersham product NXA 931), diluted 1 in 500 with PBST containing 0.5% w/v BSA, was added and the plates were incubated for 1 hour at room temperature before discarding the liquid and washing the wells twice with PBST. One hundred microlitres of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) solution, freshly prepared using one 50mg ABTS tablet (Boehringer 1204 521) in 50ml freshly prepared 50mM phosphate-citrate buffer pH5.0 + 0.03% sodium perborate (made with 1 phosphate citrate buffer with sodium perborate (PCSB) capsule (Sigma P4922) per 100ml distilled water), was added to each well. Plates were then incubated for 20-60 minutes at room temperature until the optical density value of the "total" control wells, measured at 405nm using a plate reading spectrophotometer, was approximately 1.0. "Blank" (no ATP) and "total" (no compound) control values were used to determine the dilution range of test compound which gave 50% inhibtion of enzyme activity.

(b) In Vitro HUVEC Proliferation Assay

This assay determines the ability of a test compound to inhibit the growth factor-stimulated proliferation of human umbilical vein endothelial cells (HUVEC).

HUVEC cells were isolated in MCDB 131 (Gibco BRL) + 7.5% v/v foetal calf serum (FCS) and were plated out (at passage 2 to 8), in MCDB 131 + 2% v/v FCS + 3μg/ml heparin + 1μg/ml hydrocortisone, at a concentration of 1000 cells/well in 96 well plates. After a minimum of 4 hours they were dosed with the appropriate growth factor (i.e. VEGF 3ng/ml, EGF 3ng/ml or b-FGF 0.3ng/ml) and compound. The cultures were then incubated for 4 days at 37°C with 7.5% CO_2. On day 4 the cultures were pulsed with 1μCi/well of tritiated-thymidine (Amersham product TRA 61) and incubated for 4 hours. The cells were harvested using a 96-well plate harvester (Tomtek) and then assayed for incorporation of tritium with a Beta plate counter. Incorporation of radioactivity into cells, expressed as cpm, was used to measure inhibition of growth factor-stimulated cell proliferation by compounds.

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6. That the results obtained from the test procedures described above are shown in the Table attached hereto as APPENDIX I as follows:

Compound Identification

Column 1 lists the number of the Example in the specification of the subject application corresponding to the compound tested, and also lists C1 and C2 the examples from US 6,645,969 that were tested;

Column 2 identifies the linker Zb;

Column 3 identifies the substituent at the 4-position of the quinazoline ring;

Column 4 identifies the substituent at the 6-position of the quinazoline ring;

Column 5 identifies the substituent at the 7-position of the quinazoline ring;

Enzyme Assay - results reported as the micromolar ranges within which the IC_{50} for each compound lies, or as an accurately calculated IC_{50}. The IC_{50} is the concentration of compound which reduces the enzyme activity by half of the difference of the activity in the presence of 2μM adenosine-5'-triphosphate (ATP) and in the absence of 2μM ATP:

Columns 6 and 7 report the test results from the "Enzyme Assay" with respect to cFlt and cKDR, which are the cytoplasmic domains of vascular endothelial growth factor receptors Flt1 and KDR respectively;

HUVEC Assay - results reported as micromolar ranges within which the IC_{50} for each compound lies, specifically the concentration of compound which inhibits the proliferation, as measured by tritiated thymidine incorporation, of human umbilical vein endothelial cells (HUVEC) by half the difference between the growth factor-stimulated proliferation and the background unstimulated proliferation which occurs over the duration of the assay in the absence of dosage with growth factors.

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Column 8 reports the test results from the HUVEC Assay with respect to vascular endothelial growth factor;

Column 9 reports the "basal" concentration, which refers to the concentration of compound which inhibits the growth factor-stimulated incorporation of tritiated thymidine to a level of incorporation of tritiated thymidine below that observed with cells not treated with growth factor or compound during the course of the assay, which may indicate non-specific effects of compound on DNA synthesis.

7. That from the *in vitro* data provided in the Table of APPENDIX I for compounds of the invention exemplified in the subject application he believes that the following conclusions can reasonably be drawn:

(a) With respect to the Enzyme Assay

the data indicate that substantially all of the tested compounds possess significant activity *in vitro* as inhibitors of the tyrosine kinase activity of the VEGF receptors KDR and/or Flt1, in contrast to the compounds C1 and C2 of US Patent No. 6,645,969 which possess no significant activity *in vitro* as inhibitors of either VEGF receptor KDR or Flt1;

(b) The HUVEC assay allows comparison of compounds which have been shown to be active in the enzyme assays, but the HUVEC assay requires a certain level of enzyme activity in order to give meaningful data. The HUVEC assay also requires the compound to enter the cells; certain compounds cannot do this and will therefore be inactive in the HUVEC assay despite having good enzyme activity. At low activity levels (*e.g.*, an IC_{50} of 1-10 and above) the HUVEC assay is too coarse to provide meaningful quantitative data; nonetheless, taking due account of this factor, the following conclusions can reasonably be drawn:

the data indicate that over half of the compounds that were tested in the HUVEC assay show moderate to high activity as *in vitro* inhibitors of VEGF dependent

thymidine incorporation, in contrast to the compounds C1 and C2 of US Patent No. 6,645,969, where the HUVEC data qualitatively supports the conclusion that these latter compounds possess no significant activity *in vitro* as inhibitors of VEGF dependent thymidine incorporation.

9. The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and on belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and such willful false statements may jeopardise the validity of Application Serial No.09/806,836 or any patent issuing thereon.

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APPENDIX I

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9
Examples from US Patent Application No. 09/806,836					(a) Enzyme Assay		(b) HUVEC Assay	
Example No.	Linker Zb	4-position subst	6-position subst	7-position subst	cFlt	cKDR	VEGF	Basal
2	O	4-(5-O-pyrrazolyl-3-CH2Ph)	OMe	O(CH2)3-morph	0.14-1.2 (1.2-3.7 susp)	0.015-0.05 (0.14-0.4 susp)	0.1-1	>10
3	O	4-(5-O-pyrrazolyl-3-Ph)	OMe	O(CH2)3-morph	0.4 (susp)	0.015-0.05 (susp)	~0.1	1-10
4	O	4-(5-O-pyrrazolyl-3-Ph)	OMe	OCH2CH2OMe	0.4-1.2	<0.002	>1	>1
5	O	4-(5-O-pyrrazolyl-3-(4-F-Ph))	OMe	O(CH2)3-morph	1.27	0.052	0.1-0.3	1-10
6	O	4-(5-O-pyrrazolyl-3-Ph)	H	OCH2CH2OMe	1.2-3.7	0.002-0.015	>10	>10
7	O	4-(5-O-pyrrazolyl-3-Ph)	OMe	OCH2CH2-1-imidazolyl	1.2-3.7	0.05-0.14	~0.1	>0.3
8	O	4-(5-O-pyrrazolyl-3-Ph)	OMe	(OCH2CH2)2OMe	4.37	0.005-0.015	0.244	1-10
9	O	4-(5-O-pyrrazolyl-3-(3,4-diMeO-Ph))	OMe	O(CH2)3-morph	>100	3.7-11		
10	O	4-(5-O-pyrrazolyl-3-(4-MeO-Ph))	OMe	OCH2CH2OMe	>3.7	0.015-0.14	~0.1	>1
11	O	4-(5-O-pyrrazolyl-3-(3-Pyridine))	OMe	O(CH2)3-morph	3.7-11	0.14	~1-10	>10
12	O	4-(5-O-pyrrazolyl-3-(4-Cl-Ph))	OMe	O(CH2)3-morph	0.377	<0.002	0.0225	1-10
13	O	4-(5-O-pyrrazolyl-3-(4-pyridine))	OMe	O(CH2)3-morph	3.7-11	0.05-0.14	~1-10	>10
14	O	4-(5-O-pyrrazolyl-3-Ph)	OMe	O(CH2)3-piperaz-NMe	0.0632	<0.002	0.0334	1-10
23	O	4-O-(3-pyrazol-5-(3-furan))	OMe	O(CH2)3-morph	0.4-1.2	0.015-0.05	0.01-0.1	1-10
24	O	4-O-(3-pyrazol-5-(2-F-phenyl)	OMe	O(CH2)3-morph	1.2-3.7	0.05-0.14	~0.1	1-10
25	O	4-O-(3-pyrazol-5-(3-NO2-phenyl)	OMe	O(CH2)3-morph	>3.7	0.05-0.14		
26	O	4-O-(3-pyrazol-5-(4-NO2-phenyl)	OMe	O(CH2)3-morph	>3.7	0.05-0.14	~1	1-10
27	O	4-O-(3-pyrazol-5-nPr)	OMe	O(CH2)3-morph	>3.7	0.14-0.4	~1-10	>10
28	O	4-(5-O-pyrrazolyl-3-CH2CH2CH=CHMe)	OMe	O(CH2)3-morph	>3.7	0.015-0.4	~1	>10
29	O	4-O-3-pyrazol-5-CH2OMe	OMe	O(CH2)3-morph	>3.7	0.14-0.4	~1	>10
30	O	4-O-3-pyrazol-5-Et	OMe	O(CH2)3-morph	>3.7	1.2-3.7		
31	O	4-(5-O-pyrrazolyl-3-CH2CH2Ph)	OMe	O(CH2)3-morph	>3.7	1.2-3.7		
32	O	4-(5-O-pyrrazolyl-3-(4-MeO-Ph))	OMe	OCH2-4-piperidin-NMe	0.4-1.2	0.002	0.01-~0.1	5-10
33	O	4-(5-O-pyrrazolyl-3-Ph-4-MeO)	OMe	OCH2CH2-1-[1,2,3]-triazolyl	>3.7	0.015-0.05	>0.1	5-10
34	O	4-(5-O-pyrrazolyl-3-Ph-p-OMe)	OMe	OCH2-4-piperidin-N-CH2CH2SO2Me	>3.7	0.002-0.015	0.05-0.1	>10
35	O	4-(5-O-pyrrazolyl-3-Ph(4-OMe))	OMe	O(CH2)3-piperaz-NMe	0.14-0.4	0.005-0.015	0.01-0.1	1-10
36	O	4-(5-O-pyrrazolyl-3-isoBu)	OMe	O(CH2)3-morph	>3.7	0.4-1.2		
37	O	4-(5-O-pyrrazolyl-3-nBu)	OMe	O(CH2)3-morph	>3.7	0.14-0.4		
38	O	4-(5-O-pyrrazolyl-3-CH2CH2-cyclopentyl)	OMe	O(CH2)3-morph	>3.7	0.4-1.2		
39	O	4-(5-O-pyrrazolyl-3-Ph-p-MeO)	OMe	O(CH2)3SO2Me	>3.7	0.4-1.2		
C1	O	4-(3-chlorophenyl)	OMe	OMe	>33	1.6	1.1	>10
C2	O	4-(3,4,5-trimethoxyphenyl)	OMe	OMe	>100	>100	>10	>10

susp means suspension, the true IC50 of such compounds will be lower than that given for a suspension